WS14: PHOTOTOXICITY IN LIVE FLUORESCENCE IMAGING—UNDERSTANDING, QUANTIFYING, AND MINIMIZING IT

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Introduction and Aims

Modern fluorescence microscopy techniques offer an unprecedented view inside living biological specimens. However, careful execution is required, not to alter the physiology of the living samples during the observation. Excess illumination causes phototoxicity through generation of reactive oxygen species (ROS) and destruction of endogenous lightabsorbing molecules. Most living organisms have evolved mechanisms to deal with a limited amount of ROS produced in the cell. To collect correct and reproducible data, researchers must aim to avoid exhausting the capacity of these endogenous mechanisms. It remains a challenging task to detect when the phototoxicity threshold has been exceeded, as the first manifestations of phototoxicity are rather subtle. Two commonly used, albeit unreliable readouts, are sample morphology changes and fluorophore photobleaching. Even once the phototoxicity in an experiment is recognized, it can prove difficult to significantly reduce the illumination without considerably altering the microscope setup or experimental conditions. Fortunately, strategies to remedy phototoxicity exist and typically involve altering parameters of the illumination. The most effective strategy involves confining the illumination to the focal plane of the detection objective by using light sheet or total internal reflection fluorescence microscopy. Others include increased exposure time, which decreases the peak illumination intensity at the expense of lower frame rate. Pulsed rather than continuous illuminations leaves dark periods for the specimen to recover from the incurred photodamage. Shift to red and far-red fluorophores allows the use of less damaging illumination wavelengths. Optimized detection efficiency (cameras, filters, optical path) allows for lower illumination power. Antioxidants in the media help scavenge ROS.

We organized this workshop because the implications of phototoxicity for live fluorescence imaging remain widely underrated. This issue is acknowledged and has been discussed extensively among microscopists (116–121), also on the pages of Cytometry Part A (122). However, with the increased availability of advanced microscopes, the number of scientists using live imaging is growing fast, with many of them remaining unaware of the dangers of phototoxicity. In other cases, phototoxicity is largely overlooked with the fatalistic attitude that it cannot be avoided. This workshop aimed to establish and raise the level of awareness among members of the International Society for Advancement of Cytometry (ISAC) about the challenges associated with phototoxicity. First, we questioned the workshop participants to learn about the existing and perceived barriers to adopting best practices against phototoxicity (instrumentation, time, experimental protocol). Second, we wanted to understand how difficult it would be to overcome these barriers (education, cultural shift,

new instrumentation). Finally, we drew experience from the invited expert panelists and the audience to sketch current

guidelines for addressing phototoxicity in live cell imaging. The workshop was attended by a balanced mix of students, senior scientists, microscopy facility managers, as well as industry representatives, who all contributed different perspectives in a lively discussion.

Outcome

A number of key challenges that lie ahead were identified during the discussion with the panelists and participants. Overcoming them has the potential to hugely improve the current common practice in live imaging.

Challenge #1: Overcoming the lack of awareness of phototoxicity and the status quo.

In our survey, half of the participants answered that they perform live imaging on a point scanning confocal microscope, which is clearly not optimal from the phototoxicity perspective. More suitable systems are also being employed, as half of the attendees reported using wide-field and 20% light sheet and total internal reflection fluorescence (TIRF) microscopy. Additionally, 20% answered that they do not control for phototoxicity at all. One point that was raised was that even though the general awareness of phototoxicity issues might be good, it is often worse among trainees who are usually performing most of the experiments. Better training from light microscopy facility managers was identified as a key action to improve phototoxicity awareness. Newcomers to the field of live imaging need to be pointed to the recent reviews on the topic (116,117,119) and encouraged to implement the recommendations in practice in their own experiments. Microscope manufacturers were criticized for not providing realistic estimates of what type of live imaging experiments are feasible with their systems.

Challenge #2: Insufficient reporting of live imaging conditions in publications.

Having sufficient information about the live imaging experiment is essential for fair assessment of the reported data, which currently is not always possible. It would be desirable for scientific journals to adopt a policy of minimal information required for reporting live imaging experiments. Improved reporting would build awareness and eventually establish safe illumination levels for widely used model systems.

Challenge #3: Quantification of subtle phototoxicity effects not evident at the morphological level.

Two thirds of the workshop participants evaluate phototoxicity purely on morphological level, e.g. from membrane blebbing, which can easily result in underestimations (116). Metabolism itself is highly sensitive to any kind of injury including phototoxicity and metabolic changes appear much earlier than any morphological changes, for example, (123). Our panel suggested direct oxidative stress measurement, e.g. of lipid oxidation or increase in intracellular Ca^{2+} concentration, as relatively simple and very sensitive readouts (Fig. 5, references in (116)).

When imaging mammalian embryos, a more complex but relevant way to measure phototoxicity is to reimplant imaged embryos in the carrier mothers as has been done for two-photon (124) or light sheet imaging (125). Their development to pups has been followed in comparison to nonilluminated fetuses. Similarly, the Beaurepaire lab investigated the development of Drosophila embryos after excitation

between 1 and 1.2 μm (126).

Challenge #4: Lack of commonly available hardware and fluorophores optimized for imaging in the far-red spectrum.

Shifting excitation toward the red and far-red wavelengths alleviates phototoxicity and decreases background fluorescence from the absorption by endogenous molecules (116). Reducing background fluorescence should be viewed as an equally powerful strategy to improve the image contrast as is increasing the signal (121,127). There was a consensus that switching to longer wavelengths offers a considerable potential for reducing phototoxicity and indeed, half of the participants are already doing that. Still, half of the participants reported that they use GFP constructs out of habit or because their

Figure 5. Increasing intracellular concentration of Ca^{2+} during a time-lapse experiment. Although the neuronal morphology does not change, the increased Ca^{2+} concentration indicates phototoxicity. The Ca^{2+} concentration was measured by a FRET sensor TN L15 based on Troponin C, having Cerulean as the FRET donor and Citrine as the FRET acceptor. The shorter fluorescence lifetime of Cerulean indicates a higher FRET efficiency and, thus, a higher neuronal Ca^{2+}. The montage shows a brain slice from a CerTN L15 mouse, which expresses TN L15 in several neuronal subsets under repeated excitation (every 30 s) at a mean laser power of 50 mW at 850 nm; pulse width 140 fs. Image size 300×300 μm.

trusted constructs are GFP fusions, citing historical reasons or technical limitations. Most live imaging setups are optimized for GFP, as detection components have often poor efficiency in the far-red spectrum. Microscope manufacturers should reflect the needed shift toward longer wavelengths and bring appropriate live imaging setups to the market. Development of better far-red fluorophores and detectors is a field of active research and will keep providing improvements in the future.

Challenge #5: Synergistic effects of cellular stress and phototoxicity.

Phototoxicity assessment is often performed on unstressed samples, which does not reflect the usual experimental situation of suboptimal culture conditions or inhibition/knockout of important cellular proteins. For example, the capacity of cells to scavenge ROS is reduced when they are metabolically challenged. It is therefore essential to consider that the more stress the experimental conditions impose on the cells, the sooner and more likely will phototoxicity occur.

Challenge #6: Introducing the low O_2 concentration imaging.

Imaging of mammalian cells is typically performed at near-atmospheric O_2 concentration, which is much higher than its concentration inside the tissues in vivo. At the same time, it is known that lowering O_2 concentration can reduce phototoxicity and photobleaching (128,129). Despite these obvious benefits, low O_2 imaging is rarely performed. This is mainly because maintaining the cells in hypoxic conditions is technically demanding and such experiments generate data that are not directly comparable to the body of existing literature. Thus, it remains to be seen whether the live imaging field will move in the direction of low O_2 imaging.

Challenge #7: Reducing phototoxicity in multi-photon microscopy.

In the case of imaging deep inside living tissues or organisms, multi-photon microscopy remains the method of choice. In multiphoton microscopy, both the processes of photodamage and photobleaching at the focal plane follow a highly nonlinear dependency, even higher than the excitation itself (130). Reducing the repetition rate of the lasers, which enables a longer time for the cells to recover before the next

excitation (131) and pushing the excitation further to the infrared and the emission further to red or even near-infrared are suitable strategies to reduce phototoxicity (132–134).

Challenge #8: The use of image reconstruction software algorithms for enhancing image contrast needs to be streamlined.

Recent publications using deep learning for reconstruction of fluorescence microscopy images (135,136) hold huge promise for the future. Such algorithms can reconstruct high quality images from noisy raw images acquired at low illumination levels. Currently, using these algorithms is not straightforward and their implementation for reconstruction of individual raw data-sets needs to be streamlined. We expect continuing rapid development in this area, which will make these algorithms accessible to a wide community of biologists.

Perspectives

The overall atmosphere at the workshop was optimistic. Participants agreed that we no longer have to nor should accept phototoxicity in our experiments. Technological advances like light sheet microscopy, improved scientific Complementary metal-oxide-semiconductor (sCMOS) cameras, new fluorophores, and so forth (see Table 5) were

Table 5. Measures to alleviate phototoxicity in fluorescence microscopy and appropriate control experiments

ACTION	POWER TO REDUCE PHOTOTOXICITY	CHALLENGES
Imaging media additives	+	The experiment is performed in conditions different from the standard in the field, for example, in hypoxia when using oxygen scavengers
Extending exposure time and reducing illumination intensity, pulsed illumination	++	Not suitable for very fast processes
Using far-red fluorescent probes	++	Detection components have lower efficiency in that spectral range, far-red fluorophores have lower brightness
Using selective illumination, for example, light sheet	+++	Need for potentially expensive hardware and new sample mounting strategies
Using deep learning for image restoration	++	Currently requires advanced coding skills

Potential controls for phototoxicity

- Include a transmitted light channel to monitor sample morphology during imaging.
- Monitor sample health after finishing the experiment, e.g., if cells divide, or embryos hatch.
- Compare a nonilluminated to an illuminated (part of the) sample at the end of the experiment. Check for slowdown of the cell cycle (lower percentage of mitotic cells), cellular membrane blebs, delayed development of embryos, etc.
- Generate the phototoxicity (dose–response) curves (Tinevez et al, 2012; Icha et al, 2017; Schmidt et al, 2017) for novel experimental setups.

identified as the most important recent developments. Their cross-disciplinary nature however requires synergy between biologists, engineers, physicists and computer scientists. The trend of moving toward live imaging and the need for more training in image processing is also apparent from a large community survey in the recent Biotechnology and Biological Sciences Research Council (BBSRC) report on bioimaging in the United Kingdom (137). Triggering some phototoxicity should not stop us from pushing the applications at the frontier, as long as appropriate control experiments are conducted to monitor sample health (see Table 5).

In addition, increased understanding of phototoxicity mechanisms and effects can be applied beyond imaging, for example,

in photodynamic therapy or to trigger precisely localized DNA damage. Another outcome of increased awareness related to phototoxicity is that scientists and editors are becoming more rigorous and demanding when reviewing papers and judging live imaging data. This will contribute to improving reproducibility and quality of information in the literature. A refreshing suggestion from one workshop participant was that the live imaging community should crowdsource their own phototoxicity guidelines through GitHub, similar to how the biomedical deep learning community reviewed the current state of their field ((138) github.com/greenelab/deep-review). The consensus was that since the topic is so broad, several parallel guideline projects would be needed for specific fields and microscopy techniques. Additionally, there was an encouraging interest from company representatives present during the workshop. Hopefully, new light microscopy products with the purpose of controlling and reducing phototoxicity in time-lapse imaging will become available in the near future.

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